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Autoregulation of Npl3, a Yeast SR Protein, Requires a Novel Downstream Region and Serine Phosphorylation▽

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Npl3 is an SR-like protein with documented roles in mRNA export and transcription termination. Maintaining appropriate levels of Npl3 protein is critical for cell survival. Here we show that Npl3 negatively regulates its own expression via modulation of its mRNA levels. By creating gene chimeras, we demonstrate that the region downstream of the coding sequence of Npl3 is necessary and sufficient to confer regulation. The use of different polyadenylation sites in this region results in at least two stable RNAs; read-through of these sites causes the formation of 3′-extended RNAs that are highly unstable and therefore largely unproductive. Increasing the amount of Npl3 protein promotes read-through. Notably, the loss of Npl3 phosphorylation promotes the use of the productive polyadenylation sites, resulting in elevated levels of Npl3 protein. We propose that proper levels of Npl3 protein are achieved by a negative feedback loop in which phosphorylated Npl3 suppresses efficient recognition of the productive processing signals in its own transcript.

Protein-coding mRNAs are produced in the nucleus by the cotranscriptional capping, polyadenylation, and splicing of primary transcripts prior to their export to the cytoplasm and engagement with the translation machinery. In *Saccharomyces cerevisiae*, the abundant RNA-binding protein Npl3 is involved in most, if not all, of these steps. Npl3 shares structural characteristics with two large classes of RNA binding proteins in higher eukaryotes, the SR (serine/arginine-rich) and hnRNP families. Both classes contain one or more N-terminal RNA recognition motifs, while their C termini contain amino acids required for distinctive posttranslational modifications (6). SR proteins are reversibly phosphorylated on multiple SR/RS dipeptides (reviewed in references 13 and 38), while many hnRNP proteins contain dimethylated arginines within closely repeated RGG tripeptides (23). Npl3 contains two RNA recognition motifs in its N terminus, and the C-terminal domain contains a number of RGG tripeptides (six of which are in the sequence context SRGG) and two RS dipeptides (3, 30). Previous work has shown that Npl3 is both methylated and phosphorylated (10, 25, 34, 46). Unlike that of mammalian SR proteins, Npl3 phosphorylation depends on the presence of a single RS dipeptide. Notably, the Sky1 kinase has high homology to the mammalian SR-specific protein kinase SRPK (10, 33).

Mammalian SR proteins are best understood for their roles in constitutive and regulated alternative splicing (reviewed in reference 13). More recently, a role for mammalian SR proteins in mRNA export has also been described (16, 17, 20). Conversely, yeast (*S. cerevisiae*) Npl3 was first identified by its requirement in mRNA export (21, 35), but we have now shown that Npl3 is also required for the efficient splicing of a subset of intron-containing genes (T. L. Kress and C. Guthrie, unpublished data). Interestingly, a novel role for Npl3 was identified in a genetic screen for mutants in which the efficiency of transcription termination is increased (4). Recently, this function has been correlated in vitro with the ability of high levels of Npl3 to antagonize the recruitment of factors required for 3′ mRNA processing (4, 5, 44). Finally, impairment of the ability of Npl3 to shuttle results in increased association with polysomes, suggesting a role for Npl3 in the regulation of translation (43).

In light of the broad range of cellular functions with which Npl3 is associated, it is anticipated that the activity of Npl3 is tightly regulated, likely by multiple mechanisms. One way in which the activity of Npl3 is controlled is via posttranslational modification. The phosphorylation state of Npl3 is critical for its role in mRNA export, where dephosphorylation of RNA-bound Npl3 by the phosphatase Glc7 in the nucleus is coupled to the recruitment of the major mRNA export receptor Mex67 to the RNA (9, 10). Although the precise timing of dephosphorylation is unknown, Glc7 is physically associated with the 3′ end machinery (27, 42). This association parallels the situation in mammals, where hypophosphorylated SR proteins act as adaptors for the mammalian homolog of Mex67, TAP/NXF1 (17, 20). Npl3 is exported, together with the mRNA (19), and once in the cytoplasm, Npl3 is phosphorylated by the kinase Sky1, allowing for the dissociation of the newly exported Npl3/RNA complexes and the reimport of Npl3 into the nucleus by the karyopherin Mtr10 (10, 28, 32, 46).

Balanced levels of Npl3 are critical for cell viability; both the overexpression and deletion of Npl3 are toxic (7, 36), indicating that the expression of Npl3 might also be regulated. A particularly effective strategy for controlling the levels of RNA binding proteins is through autoregulation; for example, when binding to a primary RNA target approaches saturation, further gene expression can be down-regulated by binding to secondary RNA targets (in this case, the protein's own RNA). We report here that Npl3 regulates its own expression by a

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TABLE 1. Yeast strains used in this study[a]

Strain	Genotype	Source
yMKL200	*MAT***a** *LYS2*$^+$ *met15*Δ	[b]
yTK87	*MAT***a** *npl3-S*$_{411}$*A LYS2*$^+$ *met15*Δ	This study
yMKL185	*MAT***a** *NPL3-ADH1*$_{UTR}$*::HIS3 LYS2*$^+$ *met15*Δ	This study
yMKL186	*MAT***a** *npl3-S*$_{411}$*A-ADH1*$_{UTR}$*::HIS3 LYS2*$^+$ *MET15*$^+$	This study
yMKL187	*MAT***a** *NPL3-ADH1*$_{UTR}$*::HIS3 rrp6::KAN LYS2*$^+$ *MET15*$^+$	This study
yMKL201	*MAT***a** *rrp6::KAN LYS2*$^+$ *met15*Δ	[b]
yMKL252	*MAT***a** *upf1::KAN LYS2*$^+$ *met15*Δ	[b]
yMKL260	*MAT***a** *xrn1::KAN LYS2*$^+$ *met15*Δ	[b]
yMKL281	*MAT***a** *rrp6::HIS3 LYS2*$^+$ *met15*Δ	This study
yMKL283	*MAT***a** *npl3-S*$_{411}$*A rrp6::HIS3 LYS2*$^+$ *met15*Δ	This study
yMKL325	*MAT***a** *xrn1::KAN rrp6::HIS3 lys2*Δ *MET15*$^+$	This study
yMKL327	*MAT***a** *xrn1::KAN rrp6::HIS3 npl3-S*$_{411}$*A LYS2*$^+$ *MET15*$^+$	This study

[a] All yMKL *S. cerevisiae* strains are in the consortium strain background (*ura3*Δ *leu2*Δ *his3*Δ).
[b] From the consortium deletion library.

novel mechanism in which appropriate levels of Npl3 are achieved by the inhibition of its normal cleavage and polyadenylation signals. Increasing levels of Npl3 protein suppress the use of these sites, resulting in 3′-extended RNAs that are targeted for degradation. This mechanism fits well with the observation that Npl3 can prevent premature transcription termination by suppressing cryptic 3′ end formation (4, 44). Importantly, effective autoregulation requires that Npl3 be phosphorylated. We propose that phosphorylated Npl3 suppresses the use of the productive 3′ end formation signals in the 3′ untranscribed region (UTR) of *NPL3*.

MATERIALS AND METHODS

Yeast strains. The yeast strains used and constructed in this study are listed in Table 1. All strains are isogenic with yMKL200, which is in the deletion consortium genetic background (S288C derived). yTK87, where the *S*$_{411}$*A* mutation is integrated in the *NPL3* locus, was made by homologous recombination using MfeI-digested pRS306-npl-S$_{411}$A and yMKL200 as the parental strain. yMKL186, in which the chromosomal copy of *NPL3* is replaced by the *S*$_{411}$*A* mutation, followed by the *ADH1* terminator and the *His3MX6* module, was generated by the method of Longtine et al. (24) by using yMKL200 as the parental strain and primers OWG63 (5′-AGAACCAGAGATGCTCCACGTG AAAGAGCACCAACCAGGTGAGGCGCGCCACTTCTAAA-3′) and OWG54 (5′-TGTTTTCCTTTTTCATTTGTTCTCAGTCTCATATTTAAGGAATTCGA GCTCGTTTAAAC-3′). yMKL185 and yMKL187, where wild-type *NPL3* is followed by the *ADH1* terminator and the *His3MX6* module, were created using by yMKL200 and yMKL201 as the parental strains, respectively, and the same OWG54 reverse primer and OWG62 (5′-AGAACCAGAGATGCTCCACGTG AAAGATCACCAACCAGGTGAGGCGCGCCACTTCTAAA-3′). yMKL281 and yMKL283, in which the chromosomal copy of *RRP6* is replaced with the *His3MX6* module, were created by the method of Longtine et al. (24) by using yMKL200 and yTK87 as the parental strains, respectively. yMKL325 and yMKL327 were isolated from a cross between yMKL283 and a *xrn1::KAN MAT*α strain from the consortium deletion library. Yeast was grown at 30°C in yeast extract-peptone-dextrose or in selective medium (synthetic dextrose-URA or synthetic dextrose-LEU) when plasmids were present.

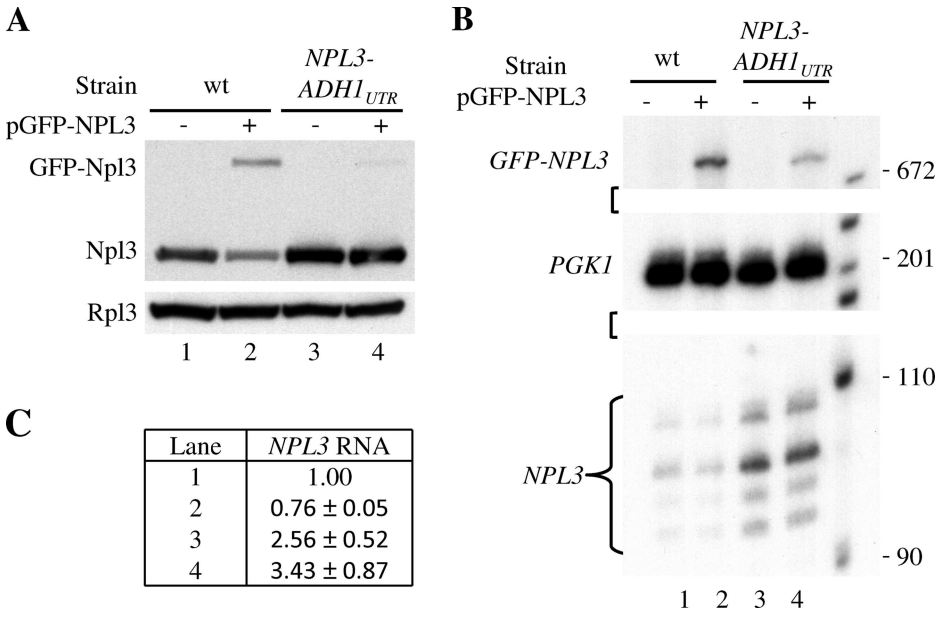
Plasmids. pRS315-GFP-Npl3 and pRS315-GFP-npl3-S$_{411}$A have previously been described (10). pRS306-npl3-S$_{411}$A was made by inserting the PvuII fragment from pRS315-GFP-npl3-S$_{411}$A that contains the open reading frame (ORF) and 3′ UTR of Npl3 into PvuII-digested pRS306. pRS316-GFP-DBP5$_{UTR}$ was obtained by homologous recombination in wild-type yeast of BsrGI-digested pRS316-Dbp5 (40) and a DNA fragment containing the coding region of green fluorescent protein (GFP) flanked by the last 40 and first 40 nucleotides (nt) of the 5′ UTR and 3′ UTR, respectively, of DBP5. The GFP-containing fragment was made by PCR using the primers OML64 (5′-CTTAC CACCTTAGATCGGAATGAGTGATACAAAGAGAGATAGTAAAGGAG AAGAACTTTT) and OML65 (5′ AAGCTTTTACGTATTTTGAGGTATTAT GTACTGAATTCTATCGCGATTTGTATAGTTCATCCATGC-3′), with pRS315-GFP-Npl3 as the template. pRS316-GFP-NPL3$_{UTR}$ was obtained by homologous recombination in wild-type yeast of NruI/BamHI-digested pRS316-GFP-DBP5$_{UTR}$ and a DNA fragment containing the 3′ UTR (nt 1 to 1136 after the ORF) of Npl3 flanked by the last 40 nt of the coding region of GFP and the first 40 nt following the BamHI site in pRS316-GFP-DBP5$_{UTR}$. The fragment was made by PCR using the primers OML68 (5′-AGCTGCTGGGATTACACATG GCATGGATGAACTATACAAATAAGCCATTTATATAGTTGA-3′) and OML69 (5′-TGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTG GGTACACACTTCAATTAGTAC-3′), with pRS315-GFP-Npl3 as the template.

RNA analysis. RNA was extracted from exponentially growing yeast cultures by the hot-phenol method (31). For 3′ rapid amplification of cDNA ends (RACE) experiments, 10 μg total RNA (purified as described above) was mixed with 5.5 μg of the adaptor primer (5′-GTTTCCCAGTCAGATCT$_{19}$V-3′) in 10 μl of annealing buffer (300 mM NaCl, 10 mM Tris [pH 7.5], 2 mM EDTA), incubated at 65°C for 10 min and transferred to ice. The hybridization mixture was transferred to 40 μl of preheated 1.25× extension mix (1.25 mM each deoxynucleoside triphosphate, 12.5 mM dithiothreitol, 12.5 mM Tris [pH 8], 7.5 mM $MgCl_2$) and incubated at 45°C for 60 min after the addition of 20 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc.). The cDNA was used in PCRs by using a reverse primer annealing to the adaptor primer (5′-G TTTCCCAGTCAGATCT-3′), in combination with either a reverse primer annealing to the ORF of *NPL3* or a reverse primer annealing to positions 236 to 255 in the 3′ UTR. The PCR products were gel purified and sequenced. Primer extension analyses were performed on 10 μg of total RNA as described previously (2), and the data were quantified by PhosphorImager analysis. For Northern blotting analysis, 8 μg of total RNA in H_2O was resuspended in denaturing loading buffer (50% formamide, 6% formaldehyde, 10% glycerol, 0.1 mg/ml ethidium bromide, and dyes) and separated in a 1.2% agarose gel (39). Gel and running buffer contained 0.5× Tris-borate-EDTA and no formaldehyde. The RNA was transferred to a HybondN+ (Amersham) membrane and UV cross-linked before the membrane was blocked for 2 h at 65°C with Rapid-Hyb buffer (Amersham). Next, the RNA was hybridized with a ^{32}P-end-labeled probe in Rapid-Hyb buffer at 42°C for 16 to 20 h. The membrane was washed two times with washing buffer (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate [SDS]) at 42°C for 10 min and three times at room temperature for 20 min. The RNA was quantified by PhosphorImager analysis. At least two independent experiments were carried out in order to confirm reproducibility of the results. The following oligonucleotides were used for the primer extension and Northern analysis: for *NPL3* and *GFP-NPL3* RNA, 5′-GGTGCTGTTCTTCGACTGGGGCATCG-3′; for *GFP* RNA, 5′-TGTGCC CATTAACATCACCA-3′; for *PGK1* RNA, 5′-ATCTTGGGTGGTGTTCC-3′; and for U2 snRNA, 5′-CAGATACTACACTTG-3′.

Protein analysis. Whole-cell lysate was prepared from equal A_{600} amounts of exponentially growing yeast cultures by bead-beating in trichloroacetic acid (TCA) buffer (20 mM Tris-HCl [pH 8.0], 50 mM NH_4OAc, 2 mM EDTA, 10% TCA) in the presence of protease inhibitors. The lysate was centrifuged, and the pellets were resuspended in TCA-Laemmli loading buffer (3.5% [wt/vol] SDS, 80 mM Tris-base, 8 mM EDTA, 14% glycerol, 120 mM dithiothreitol). The protein content was analyzed by SDS-polyacrylamide gel electrophoresis, and immunoblotting was performed with the following antibodies: anti-Npl3 (1:3,000) (34), anti-Rpl3 (1:1,000) (41), and anti-GFP (1:1,000) (Roche).

RESULTS

Npl3 negatively regulates its own expression through modulation of *NPL3* RNA levels. To explore the hypothesis that Npl3 is autoregulated, wild-type yeasts were transformed with a plasmid expressing N-terminal, GFP-tagged Npl3. The GFP-Npl3 module was flanked by the *NPL3* gene's own 5′ and 3′ sequences. The downstream sequence (referred to from here on as the "3′ region" of Npl3) included the first 1,135 nt of the 1,210 nt that separate the chromosomal Npl3 ORF and the downstream ORF. In the event of autoregulation, the expectation would be that the amount of endogenous Npl3 should



C

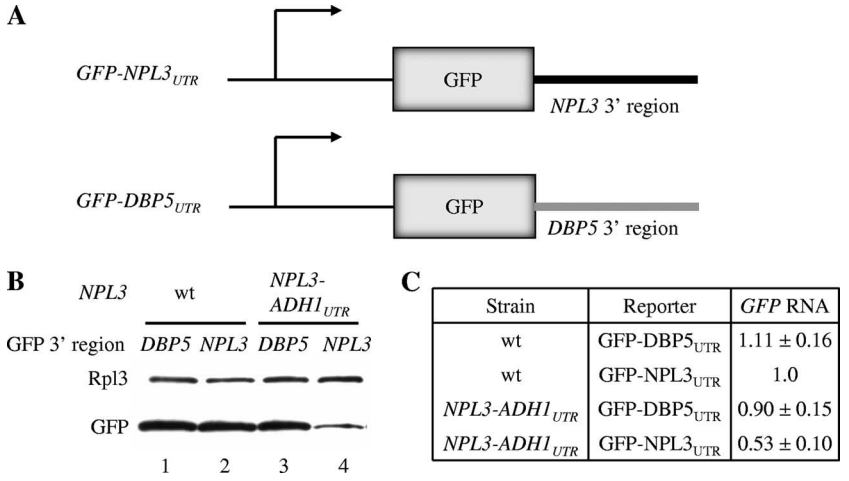
Lane	*NPL3* RNA
1	1.00
2	0.76 ± 0.05
3	2.56 ± 0.52
4	3.43 ± 0.87

FIG. 1. Npl3 is autoregulated. Whole-cell extracts were prepared from cells where the endogenous *NPL3* ORF was followed by the 3′ region of *NPL3* (wt) or *ADH1* (*NPL3-ADH1*$_{UTR}$) and contained either an N-terminally tagged GFP-Npl3 plasmid (+) or an empty vector control (−). (A) Protein content was analyzed by Western blotting using polyclonal anti-Npl3 antibodies that recognize both endogenous untagged and plasmid-borne GFP-tagged Npl3. The Rpl3 component of the large ribosomal subunit was included as an internal loading control. (B) RNA content was analyzed by primer extension using a primer hybridizing to the coding region of *NPL3* RNA, thus recognizing both endogenous untagged and plasmid-encoded *GFP-NPL3* RNA. The primer extension shown is a representative of three independent experiments. Only the sections (from the same exposure) containing primer extension products are shown. (C) The relative amounts of *NPL3* RNA were calculated after standardization to the amount of *PGK1* mRNA (average ± standard deviation of three experiments).

decrease to maintain total Npl3 at the normal level. Indeed, when Npl3 levels were examined by Western blotting, the presence of exogenous GFP-Npl3 reduced endogenous Npl3 expression (Fig. 1A, compare lanes 1 and 2). We also observed that Npl3 protein levels increased (Fig. 1A, lane 3) when the 3′ region of the endogenous *NPL3* gene was replaced with the *ADH1* terminator (referred to as *NPL3-ADH1*$_{UTR}$) as a result of PCR-mediated protein-tagging strategies. This observation strongly suggested that the 3′ region is important for maintaining an appropriate Npl3 concentration (see below). In addition, the *NPL3-ADH1*$_{UTR}$ strain provided a useful tool to test whether endogenous Npl3 can influence the level to which exogenous Npl3 can be expressed. In the *NPL3-ADH1*$_{UTR}$ strain, which has increased endogenous Npl3 levels, the amount of exogenously expressed GFP-Npl3 was reduced (Fig. 1A, compare lanes 2 and 4, and see Fig. 5A, lanes 2 and 5). Primer extension confirmed the presence of the GFP-Npl3 plasmid in the *NPL3-ADH1*$_{UTR}$ strain (Fig. 1B, lanes 2 and 4). Therefore, Npl3 negatively regulates its own expression.

Since Npl3 is an RNA binding protein with functions in multiple aspects of mRNA processing, it seemed plausible that autoregulation would happen at the level of RNA. To determine whether changes in Npl3 protein levels were mirrored by similar changes in *NPL3* RNA levels, primer extension was carried out with a primer hybridizing to nt 51 to 76 of the coding region of Npl3, thus recognizing both *NPL3* and *GFP-NPL3* RNA species (Fig. 1B). Visualization of expressed RNA using tiling microarrays predicts that transcription of the *NPL3* gene is initiated approximately 25 nt upstream of the ORF (37). In agreement with this prediction, primer extension on *NPL3* RNA resulted in multiple bands with lengths of 100 to 105 nt (Fig. 1B). This result corresponds to a 20- to 25-nt-long 5′ UTR. Consistent with the changes in protein levels, the amount of endogenous *NPL3* RNA was reduced in the presence of exogenously expressed GFP-Npl3 (Fig. 1B and C, lanes 1 and 2). In addition, the increased levels of endogenous Npl3 in the *NPL3-ADH1*$_{UTR}$ strain reduced the RNA levels of exogenous *GFP-NPL3* (Fig. 1B, compare lanes 2 and 4). Importantly, hybrid transcripts containing the *NPL3* ORF and the *ADH1* 3′ region were insensitive to exogenously expressed GFP-Npl3 (Fig. 1B and C, lanes 3 and 4), suggesting that the wild-type 3′ region of *NPL3* mediates the autoregulation by controlling *NPL3* RNA levels.

***NPL3* 3′ region can confer Npl3 responsiveness on an unrelated reporter.** The 3′ region of *NPL3* is necessary for maintaining wild-type levels of *NPL3* RNA and protein (Fig. 1A and B, lanes 3 and 4). Furthermore, the GFP-Npl3 construct that includes nt 1 to 1135 of this downstream region contains all the sequences essential for regulation (Fig. 1). To test whether the presence of the *NPL3* 3′ region would be sufficient to render an unrelated RNA and protein sensitive to the concentration of Npl3, wild-type and *NPL3-ADH1*$_{UTR}$ strains were transformed with a plasmid containing the coding region of *GFP* fused to nt 1 to 1135 of the *NPL3* 3′ region (*GFP-NPL3*$_{UTR}$) (Fig. 2A) and the expression of the GFP reporter was assayed by Western blotting (Fig. 2B). If the 3′ region of Npl3 is sufficient for autoregulation of Npl3, we would predict that the increased concentration of Npl3 in the *NPL3-ADH1*$_{UTR}$ strain should reduce GFP expression from the plasmid. Indeed, the amount of GFP produced from the *GFP-*



Strain	Reporter	*GFP* RNA
wt	GFP-DBP5$_{UTR}$	1.11 ± 0.16
wt	GFP-NPL3$_{UTR}$	1.0
NPL3-ADH1$_{UTR}$	GFP-DBP5$_{UTR}$	0.90 ± 0.15
NPL3-ADH1$_{UTR}$	GFP-NPL3$_{UTR}$	0.53 ± 0.10

FIG. 2. *NPL3* 3′ region is sufficient for regulation. (A) Yeast was transformed with reporter plasmids containing the coding region of GFP fused to the 3′ region of either *NPL3* or *DBP5*. (B and C) Whole-cell extracts were prepared from cells where the endogenous *NPL3* ORF was followed by the *NPL3* (wt) or *ADH1* 3′ region (*NPL3-ADH1*$_{UTR}$) and contained the reporter plasmids. GFP protein was analyzed by Western blotting with Rpl3 as an internal loading control (B), and RNA content was analyzed by primer extension (C). The relative amounts of *NPL3* RNA (average ± standard deviation of four independent experiments) were calculated after being standardized to the amount of *PGK1* mRNA. wt, wild type.

NPL3$_{UTR}$ reporter was dramatically reduced in the presence of the higher Npl3 concentration (Fig. 2B, compare lanes 2 and 4). To confirm that this was a specific feature of the *NPL3* 3′ region, we transformed the wild-type and *NPL3-ADH1*$_{UTR}$ strains with a plasmid containing *GFP* fused to the 3′ region of *DBP5* (*GFP-DBP5*$_{UTR}$). The amount of GFP produced from the *GFP-DBP5*$_{UTR}$ reporter was unaffected (Fig. 2B, lanes 1 and 3), indicating that the observed regulation was controlled by the *NPL3* 3′ region. Npl3 levels did not affect Dbp5 expression (data not shown). To confirm that Npl3 controls the protein concentration by influencing RNA levels, we performed primer extension on *GFP* RNA and, as predicted, the lower level of GFP protein in the *NPL3-ADH1*$_{UTR}$ strain was reflected by reduced *GFP* RNA levels (Fig. 2C; the reduction in *GFP* RNA appeared to be smaller than the reduction in GFP protein [see Discussion]). Thus, the 3′ region of *NPL3* is sufficient to confer sensitivity to Npl3 protein levels on an unrelated RNA and its corresponding protein.

Autoregulation involves production of 3′-extended *NPL3* RNA. Our results indicate that the 3′ region of *NPL3* is necessary and sufficient for Npl3 autoregulation. Because Npl3 has been shown to function as an antagonist of transcription termination/3′ end formation (4), an appealing model is that the regulation of 3′ end formation is also involved in the autoregulatory process. If Npl3 prevents the efficient termination of its own transcript, this may result in 3′-extended *NPL3* RNAs. To evaluate whether this is the case, we determined the cleavage/polyadenylation sites of the *NPL3* mRNA by using the 3′ RACE technique and identified three termination sites. 3′ RACE analysis with a forward PCR primer in the ORF produced mainly a short product (3′ RACE product 1), resulting from polyadenylation 17 nt downstream of the ORF (Fig. 3A), and a minor product (product 2), resulting from polyadenylation approximately 230 nt downstream of the ORF. Position 17 is followed by a stretch of 17 purines, primarily adenines; thus, the short product likely resulted from the hybridization of the oligo(dT) reverse transcriptase primer independently of a poly(A) tail. In addition, we detected another major site 407 nt downstream of the ORF and a minor site 449 nt downstream of the ORF (products 3 and 4) using a forward PCR primer that hybridized downstream of the 230 site.

To determine whether 3′-extended *NPL3* RNAs are in fact produced, we prepared total RNA from wild-type cells and carried out Northern blotting to analyze *NPL3* mRNA length. RNAs produced by polyadenylation at 230 and 407/449 nt downstream of the 1,245-nt-long ORF and containing a 5′ UTR of 20 to 25 nt were expected to be approximately 1,500 and 1,700 nt long, respectively (assuming that RNAs using the 407 and the 449 sites would not be separated on the gel). In agreement with this expectation, a probe annealing to the coding region detected two *NPL3* RNA species migrating just below the 1,789-nt-long 16S rRNA in a wild-type strain that were absent in a strain deleted for the *NPL3* gene (Fig. 3B, lanes 1 and 2). A probe hybridizing to position 326 to 345 downstream of the ORF recognized only the longest product (data not shown), confirming that the shorter RNA does not contain this region. Thus, the two bands migrating below the 16S rRNA most likely corresponded to *NPL3* RNAs that were cleaved and polyadenylated at positions 230 and 407/449. In addition to these RNAs, a 3′-extended RNA was present at low levels.

Because 3′-extended RNAs might be unstable and therefore difficult to detect in a wild-type strain, we carried out the assay with a number of strains deleted for factors functioning in nuclear and cytoplasmic RNA degradation and compared these results to those from the wild-type strain (Fig. 3B, lanes 3 to 5). Upon deletion of the nuclear exosome component *RRP6* or the cytoplasmic 5′-3′ exonuclease *XRN1*, the amount of 3′-extended RNA increased substantially (Fig. 3B, compare lanes 3 and 4 to lane 2). An aberrantly extended 3′ UTR can also trigger nonsense-mediated decay (NMD) (26). The 3′-extended RNA, however, was not detectably stabilized in a strain deleted for the NMD factor

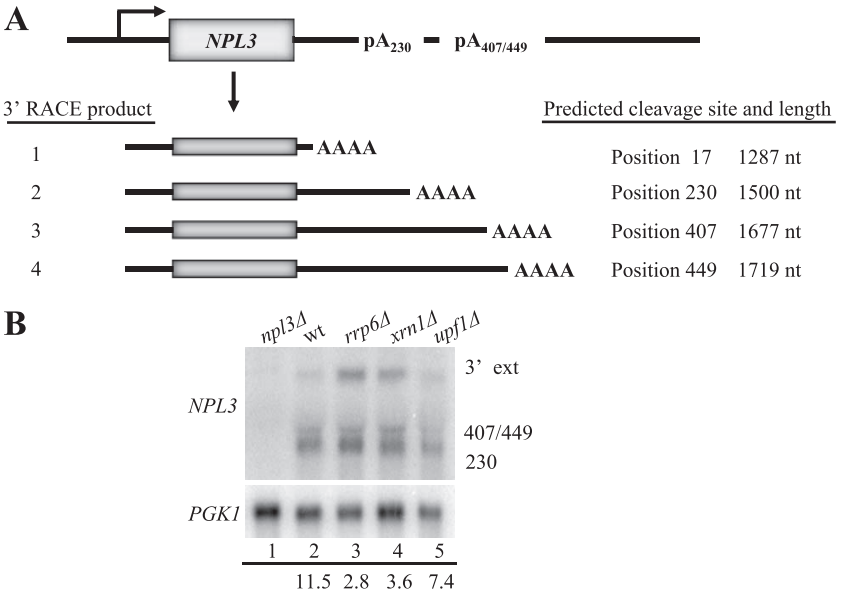


FIG. 3. Npl3 affects the balance between stable and unstable *NPL3* RNA species. (A) Schematic of cleavage/polyadenylation sites of Npl3. Shown are four potential polyadenylation sites at positions 17, 230, 407, and 449 after the ORFs were detected by 3′ RACE. Position 17 reflects the mispriming of the $dT_{20}V$ primer to positions 18 to 34 (GAAAAAAAAAGGAGAAA). (B) Whole-cell extracts were prepared from wild-type cells and cells deleted for *NPL3*, *RRP6*, *XRN1*, or *UPF1*. *NPL3* and *PGK1* RNAs were detected by Northern blotting. The ratios of the stable *NPL3* RNA species (230 and 407/449) to the 3′-extended RNA were calculated and are displayed below the lane numbers. wt, wild type; 3′-ext, 3′-extended.

UPF1 (Fig. 3B, lane 5), making it unlikely that the 3′-extended RNA is an NMD target.

If Npl3 does regulate its own expression by promoting read-through/preventing termination, we predict increased Npl3 levels to reduce the amount of the short productive RNA and increase the amount of the nonproductive 3′-extended RNA. To test this prediction, we used the *GFP-NPL3*$_{UTR}$ reporter that produces transcripts with the coding region of GFP, followed by the 3′ region (nt 1 to 1135) of *NPL3*. 3′ RACE identified cleavage/polyadenylation sites at positions 407 and 449 (data not shown), which is similar to the situation found for *NPL3* RNA. Thus, the reporter appeared to recapitulate the termination pattern of *NPL3* transcripts. This result was confirmed when we transformed both a wild-type strain that has normal levels of endogenous Npl3 and the strain that overexpresses Npl3 (*NPL3-ADH1*$_{UTR}$) with the *GFP-NPL3*$_{UTR}$ plasmid and measured the levels of *GFP* RNA species by Northern blotting (Fig. 4). While one short RNA (polyadenylation site 230) was predominant in wild-type cells, a minor RNA (polyadenylation site 407/449) and small amounts of a 3′-extended RNA were also present (Fig. 4, lane 1). As predicted, in a strain that overexpressed Npl3 (*NPL3-ADH1*$_{UTR}$), the levels of the short *GFP* RNAs were greatly reduced (Fig. 4, lane 2). We did not observe an increase in the amount of the extended RNA in *NPL3-ADH1*$_{UTR}$ cells, most likely because the RNA is unstable, as we have shown above (Fig. 3). In order to stabilize the 3′-extended RNA, we deleted the nuclear exosome component *RRP6* in the strain overexpressing Npl3 (*NPL3-ADH1*$_{UTR}$). Indeed, when *RRP6* was deleted, we observed an increase in the 3′-extended RNA (Fig. 4, compare lanes 3 and 4). These results show that excess Npl3 does indeed lead to increased formation of 3′-extended RNA, consistent with the model that Npl3 promotes read-through of the termination sites of its own 3′ region, leading to the production of an unstable, nonproductive 3′-extended RNA.

Phosphorylation of Npl3 is critical for proper regulation. The C-terminal region of Npl3 contains a serine residue (S_{411}) that is subject to phosphorylation by the cytoplasmic kinase Sky1 and dephosphorylation by the nuclear phosphatase Glc7 (9, 10). Npl3 contains eight SR/RS dipeptides in the C-terminal region, but Npl3 phosphorylation by Sky1 depends on the presence of only S_{411} (10). Furthermore, this serine is the substrate not only of Sky1 but also of additional kinases (10), strongly supporting the notion that phosphorylation is the major role of this residue. The mutation of S_{411} to alanine (*npl3-S*$_{411}$*A*) inhibits the phosphorylation of Npl3 and gives rise to

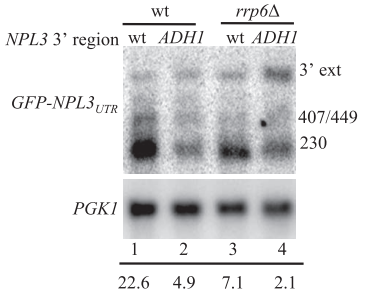


FIG. 4. Increased Npl3 levels result in an increase in unstable 3′-extended (3′-ext) *NPL3* RNA. Whole-cell extracts were prepared from wild-type, *NPL3-ADH1*$_{UTR}$, *rrp6*Δ, and *rrp6*Δ *NPL3-ADH1*$_{UTR}$ strains containing the GFP reporter with the *NPL*3 3′ region. *GFP* and *PGK1* RNAs were detected by Northern blotting. The ratios of the stable *GFP* RNA species (230 and 407/449) to the 3′-extended RNA were calculated and are displayed below the lane numbers. wt, wild type.

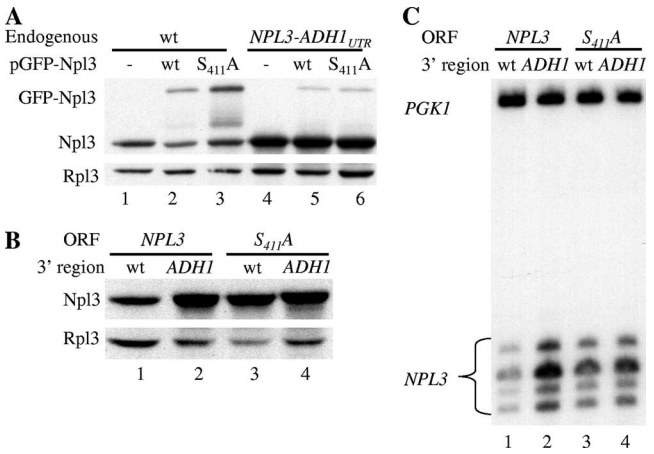
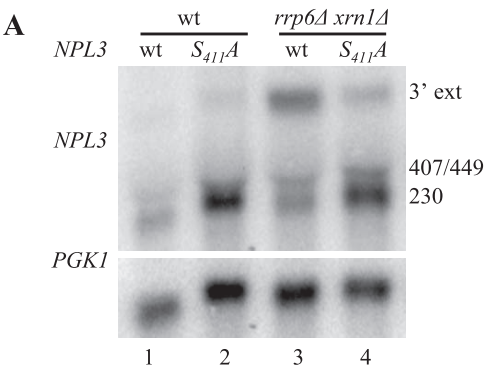


FIG. 5. Phosphorylation of Npl3 is necessary for regulation. (A) Whole-cell extracts were prepared from cells where the endogenous *NPL3* ORF was followed by the *NPL3* or *ADH1* 3′ region and contained either a wild-type (wt) GFP-Npl3 (lanes 2 and 5), a mutant GFP-S_{411}A plasmid (lanes 3 and 6), or an empty vector control (lanes 1 and 4). Protein content was analyzed by Western blotting with anti-Npl3 polyclonal antibodies recognizing both tagged and untagged Npl3. (B and C) Whole-cell extracts were prepared from cells where the coding region of wild-type *NPL3* and mutant *npl3-$S_{411}A$* was followed by the *NPL3* or *ADH1* 3′ region. Npl3 protein was analyzed by Western blotting with Rpl3 as an internal loading control (B) and RNA content was analyzed by primer extension (C). The primer extension shown is representative of four independent experiments. The relative amounts of *NPL3* RNA after standardization to the amount of *PGK1* mRNA (average ± standard deviation) were for lane 1, 1.0 ± 0.0; for lane 2, 2.16 ± 0.36; for lane 3, 1.68 ± 0.21; and for lane 4, 1.89 ± 0.22.



B

Lane	230 nt	407/449 nt	3' extended
1	1.0	0.61	0.08
2	1.97	1.23	0.17
3	1.01	0.86	1.35
4	3.02	1.91	0..57

FIG. 6. Unphosphorylated Npl3 promotes termination. Whole-cell extracts were prepared from wild-type, *npl3-$S_{411}A$*, *rrp6*Δ *xrn1*Δ, and *rrp6*Δ *xrn1*Δ *npl3-$S_{411}A$* strains. (A) RNA content was analyzed by Northern blotting. (B) The relative amounts of Npl3 RNA were calculated after being standardized to the amount of *PGK1* mRNA.

defects in mRNA export. Since the phosphorylation of Npl3 is important for at least one biological function of Npl3, we predicted that phosphorylation might also be critical for its regulation. Wild-type yeasts were transformed with plasmids expressing N-terminally tagged GFP-Npl3 and GFP-Npl3-S_{411}A. While exogenously expressed GFP-Npl3 decreased the expression levels of endogenous Npl3 modestly (Fig. 5A, lanes 1 and 2), endogenous Npl3 levels were not affected by the presence of exogenous GFP-Npl3-S_{411}A protein, indicating that phosphorylation is indeed necessary for Npl3 autoregulation. Since GFP-Npl3-S_{411}A protein is detectable in both the cytoplasm and the nucleus, while GFP-Npl3 localizes exclusively to the nucleus (10), phosphorylation could potentially be required indirectly for autoregulation due to the effect on localization. There was, however, at least threefold more GFP-Npl3-S_{411}A protein than GFP-Npl3 protein expressed from the plasmid (Fig. 5A, compare lanes 2 and 3), and even with a substantial fraction of the mutant protein in the cytoplasm, a large amount of mutant protein would be present in the nucleus. Therefore, the effect of phosphorylation on autoregulation is likely to be direct. Significantly more mutant GFP-Npl3-S_{411}A protein than wild-type GFP-Npl3 was produced from the plasmid, even though more endogenously expressed Npl3 was present (Fig. 5A, lanes 2 and 3), raising the possibility that *npl3-$S_{411}A$* RNA itself cannot be regulated. However, when the *NPL3-ADH_{UTR}* strain that overexpresses endogenous Npl3 was transformed with the GFP-Npl3-S_{411}A plasmid, the amount of exogenous GFP-S_{411}A protein was reduced (Fig. 5A, compare lanes 3 and 6), suggesting that *npl3-$S_{411}A$* RNA is sensitive to Npl3 levels. An alternative explanation for the increased expression of mutant GFP-Npl3-S_{411}A protein is that phosphorylated Npl3 is the form of the protein that that suppresses the 3′ end formation. If the lack of regulation by the Npl3-S_{411}A protein is indeed caused by a failure of the dephosphorylated protein to perform a function necessary for regulation, the phosphorylation-deficient Npl3-S_{411}A protein should also fail to downregulate its own expression when present as the only copy of *NPL3*, with the consequence being an increased concentration of mutant Npl3. Western blotting (Fig. 5B) and primer extension (Fig. 5C) confirmed that the *npl3-$S_{411}A$* strain contained both more Npl3 protein and RNA than did the corresponding wild-type strain (Fig. 5B and C, compare lanes 1 and 3). Combining the S_{411}A mutation with the *ADH1* terminator did not increase Npl3 protein and RNA levels further (Fig. 5B and C, compare lanes 2 to 4), indicating that the phosphorylation of Npl3 and the 3′ region of *NPL3* cooperate to control the concentration of Npl3 protein.

If the phosphorylation of Npl3 is required in order for Npl3 to function as an antiterminator of its own transcript, the inhibition of phosphorylation should result in the increased production of short translatable RNAs and a concomitant reduction in read-through product. Northern blot analysis showed that the levels of the short 230 and 407/449 RNAs were increased in the *npl3-$S_{411}A$* strain (Fig. 6, compare lanes 1 and 2). Since the amount of the 3′-extended RNA was almost at background levels in the wild-type strain, it was not possible to accurately detect a reduction in the *npl3-$S_{411}A$* strain. Therefore, the *npl3-$S_{411}A$* mutation was combined with the deletion of both *RRP6* and *XRN1*. The rationale for using this strain background, where both nuclear and cytoplasmic degradation is prevented, is that the *npl3-$S_{411}A$* mutation results in defects in mRNA export and therefore might affect the export of the

different Npl3 RNA species differentially. In the *rrp6*Δ *xrn1*Δ strain background, the *npl3-S411A* mutation did lead to a reduction in the production of the 3′-extended RNA and an increase in 230 and 407/449 RNAs (Fig. 6, lanes 3 and 4). Thus, phosphorylation is critical for the ability of Npl3 to prevent efficient 3′ end processing of its own transcript.

DISCUSSION

Npl3 is a highly abundant RNA binding protein whose levels must be strictly controlled; either too little or too much Npl3 is toxic (7, 36). We show here that one homeostatic mechanism involves an autoregulatory feedback loop that relies on a novel 3′ region containing several potential cleavage/polyadenylation sites. We have found that the *NPL3* gene produces at least three RNA transcripts. The use of different cleavage/polyadenylation sites results in the production of at least two stable RNAs. When mRNA degradation was inhibited by mutation of the nuclear or cytoplasmic turnover machinery, we observed the accumulation of a 3′-extended RNA. Thus, the read-through products are normally unstable and would not substantially contribute to Npl3 protein production. Notably, the 3′ region of Npl3 is both necessary and sufficient to render an unrelated GFP-reporter construct responsive to the concentration of Npl3 in vivo. That is, increased levels of Npl3 protein result in increased read-through of the normal, upstream termination/polyadenylation site(s), reducing the production of translatable transcripts and GFP reporter protein. Finally, we show that this feedback mechanism requires that Npl3 protein is phosphorylatable.

Molecular mechanism of Npl3 autoregulation. The formation of mRNA 3′ ends in yeast is a two-step procedure. The nascent transcript is cleaved 20 to 30 nt downstream of the polyadenylation signal through the cooperation of cleavage factors (CF) IA and IB and the cleavage/polyadenylation factor, followed by poly(A) tail addition by Pap1 (29). Yeast termination/polyadenylation signals are degenerate, and antitermination factors may associate with the elongating polymerase to prevent the recognition of cryptic sites located in the coding sequence. It has been suggested that Npl3 functions as such an antiterminator by reducing recruitment of the multisubunit factor CF IA (Pcf11, Rna14, Rna15, and Clp1) to weak sites (4), and an increasing amount of data supports this notion. First, *NPL3* interacts genetically with *RNA15* and the CF IB component *HRP1* (15). Second, Npl3 reduces the transcription termination efficiency of reporter transcripts both in vitro and in vivo in a way that is correlated with reduced recruitment of Rna15 to the polyadenylation site in vivo (4). In a manner similar to that described above, the binding of Npl3 to a model substrate in vitro antagonizes Rna15 recruitment (5) and, finally, Npl3 reduces use of cryptic poly(A) sites in the coding sequence of *RNA14* by blocking Rna15 and Pcf11 recruitment (44). Our data are consistent with a model in which the 3′ region of *NPL3* is sensitive to the concentration of Npl3, functioning as a natural substrate for transcription termination/3′ end formation antagonizing activity of Npl3. Thus, the general role of Npl3 as a suppressor of cryptic sites is coupled to a specific role in its own regulation. It is likely that Npl3 autoregulation is accomplished through a major activity of Npl3 and, therefore, the role of Npl3 in 3′ end formation may be more important than previously anticipated.

In mammalian cells, the U1 snRNP-specific U1A protein negatively autoregulates its own polyadenylation. U1A protein is recruited to an element in the 3′ UTR of the U1A gene, allowing U1A to interact with and inhibit the activity of poly(A) polymerase (1, 14). An appealing model is that the autoregulation of Npl3, an RNA binding protein, also involves direct binding of Npl3 to its own 3′ region. The identification of the predicted binding site, however, may be a challenging task. Chromatin immunoprecipitation experiments indicate that Npl3 is recruited to transcribed genes independently of the sequence of the nascent RNA (22), and analysis of cellular RNAs immunoprecipitated together with Npl3 failed to identify strong consensus RNA motifs correlated with Npl3 binding (18). Thus, other factors may contribute to the recruitment/binding of Npl3 to its target RNA.

***NPL3* 3′ UTR is unusual with respect to length and sequence.** The distance between *NPL3's* coding region and termination sites is unusually long (230 to 449 nt); the average 3′ UTR in *S. cerevisiae* is approximately 100 nt long (11). This might reflect inefficient termination/polyadenylation, which in turn is likely to contribute to regulation. To ask whether the use of the distal sites is simply due to the absence of suitable sites closer to the coding region, we submitted a sequence containing the *NPL3* ORF and the 1,000 downstream nucleotides to the mRNA 3′ processing site predictor (12), a discrete state-space model (DSM) that calculates the likelihood of each position being the 3′ end of the transcript. Interestingly, the DSM predictor suggested a number of high-scoring sites within 130 nt after the coding region, implying a more complex explanation. In agreement with our Northern blotting results, the DSM predictor also suggested the presence of strong 3′ end processing sites 231 and 407 nt downstream of the coding region of Npl3. The production of the 3′-extended *NPL3* RNA, however, suggests that these apparently strong sites are inefficiently used. The DSM predictor does not take the sequence context into account and occasionally makes false predictions in regions where matches to the processing elements are strong but the context for cleavage is poor (12). Thus, inhibitory sequence/structure elements within the *NPL3* 3′ UTR may antagonize the usage of otherwise strong 3′ processing signals. This is consistent with the model that Npl3 autoregulation relies on inefficient upstream 3′ end formation and an unproductive downstream site.

Additional modes of Npl3 regulation. The mutation of the Sen1 helicase, which functions in the termination of noncoding RNA polymerase II transcripts, results in a reduced association of RNA polymerase II with the 3′ end of the *NPL3* gene 3′ UTR (37). Therefore, we considered the possibility that Sen1 functions, together with Npl3, in suppressing the 3′ end processing of *NPL3* transcripts under wild-type conditions. However, we found that the mutation of *SEN1* did not affect the distribution of *NPL3* RNA species (data not shown) and, therefore, Sen1 is unlikely to be involved in Npl3 autoregulation. Future studies should address which additional factors are important for the autoregulatory mechanism.

As reviewed in the Introduction, Npl3 is an RNA binding protein predicted to be involved in an extensive range of cellular functions. The abundance of the majority of yeast pro-

teins is predicted to range from 1,000 to 10,000 molecules per cell, and so with approximately 80,000 Npl3 molecules per cell, Npl3 is a highly expressed protein (8). Given the multitude of processes that can be affected by Npl3 and its high levels, it is likely that multiple mechanisms are involved in maintaining appropriate Npl3 levels. We noted that changes in protein levels in some instances appeared larger than the accompanying change in RNA levels. In particular, the reduction in GFP protein produced from the construct expressing GFP fused to the *NPL3* 3′ region (*GFP-NPL3*$_{UTR}$) in cells overexpressing Npl3 appeared to be more dramatic than the corresponding reduction in RNA levels (Fig. 2). The association of Npl3 with translating ribosomes increases when reimport to the nucleus is compromised, suggesting a potential role in translational regulation (43). Our data are consistent with the notion that Npl3 autoregulation includes an additional layer of regulation involving negative translational control.

An additional role for posttranslational modification. Npl3 differs from typical mammalian SR proteins in two significant respects, both of which involve the status of posttranslational modifications. First, while the RS domains of SR proteins are highly phosphorylated, Npl3 phosphorylation depends on the presence of a single serine residue, S_{411}, in the so-called SR domain (10). Second, this C-terminal domain of Npl3 also contains multiple RGG repeats that are arginine methylated, a characteristic of hnRNP proteins (23, 34, 46). Since, in at least some instances, hnRNP proteins function to antagonize SR proteins in the regulation of alternative splicing, it has always been a curiosity that the yeast protein carries both modifications (see reference 34). Here we have shown that the Npl3-S_{411}A mutant protein, which cannot be phosphorylated, fails to be efficiently autoregulated, resulting in elevated levels of Npl3 protein. Our results suggest that phosphorylated Npl3 is the form of the protein that suppresses the use of the productive cleavage/polyadenylation sites in its own 3′ region and, by extension, functions generally in the suppression of cryptic 3′ sites located in coding regions of other genes. An interesting question for the future is whether methylation, which is known to promote efficient export of Npl3 (25, 45), is also required for its role in transcription termination/autoregulation. Notably, mutations that prevent the methylation of Npl3 have severe growth defects when combined with a deletion of the *CBC80* gene, which encodes a subunit of the nuclear cap binding complex (25). This observation, combined with the finding that Cbc80 plays a role in the suppression of cryptic termination/3′ end processing sites (44), points to a role for Npl3 methylation in transcription termination/autoregulation.

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